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regulatory pathway in T cell-mediated immune responses? Transplantation 1995; 60: 221.
28. Ahmed KR, Guo TB, Gaal KK. Islet rejection in perforin-deficient mice: the role of perforin and Fas. Transplantation 1997; 63: 951.
29. Seino K, Kayagaki N, Bashuda H, Okumura K, Yagita H. Contribution of Fas ligand to cardiac allograft rejection. Int Immunol 1996; 8: 1347.
30. Khan Q, Penninger JM, Yang L, Marra LE, Kozieradzki I, Zhang L. Regulation of apoptosis in mature alphabeta+CD4-CD8- antigen-specific suppressor T cell clones. J Immunol 1999; 162: 5860.
31. Djamali A, Odorico JS. Fas-mediated cytotoxicity is not required for rejection of murine nonvascularized heterotopic cardiac allografts. Transplantation 1998; 66: 1793.
32. Ito A, Minagawa M, Tomiyama K, Ito M, Kawai K. Cytotoxic pathways in the skin allograft rejection by CD4+ T cells. Transplantation 1999; 68: 97.
33. Weintraub JP, Godfrey V, Wolthusen PA, Cheek RL, Eisenberg RA, Cohen PL. Immunological and pathological consequences of mutations in both Fas and Fas ligand. Cell Immunol 1998; 186: 8.
34. Adachi M, Suematsu S, Kondo T, et al. Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. Nat Genet 1995; 11: 294.
35. Ware CF, VanArsdale TL, Crowe PD, Browning JL. The ligands and receptors of the lymphotoxin system. Curr Top Microbiol Immunol 1995; 198: 175.
36. Van Parijs L, Abbas AK. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. Science 1998; 280: 243.
37. Honey K, Cobbold SP, Waldmann H. Dominant tolerance and linked suppression induced by therapeutic antibodies do not depend on Fas-FasL interactions. Transplantation 2000; 69: 1683.

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REPROGRAMMING OF GENE EXPRESSION IN CULTURED CARDIOMYOCYTES AND IN EXPLANTED HEARTS BY THE MYOSIN ATPASE INHIBITOR BUTANEDIONE MONOXIME

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Background. **Butanedione monoxime (BDM) is a reversible myosin ATPase inhibitor. Its use in transplantation medicine may be of benefit in the preservation of hearts. As little is known about its ability to prevent stress and metabolic deregulation, we wanted to investigate the genomic response in cultured cardiomyocytes and explanted, preserved hearts at the transcriptional level.**

Methods. **We thus investigated the gene expression of the transcription factors GATA-4, Nkx2.5, MEF-2c, and Oct-1 and of the downstream target genes atrial and brain natriuretic peptide, α- and β-myosin heavy chain, α-cardiac actin, and α-skeletal actin. Additionally, lactate dehydrogenase and creatine kinase enzyme activities were measured as markers for membrane integrity and metabolic deregulation of cardiomyocytes.**

Results. **In untreated cardiomyocyte cultures, expression of GATA-4 and Nkx2.5 was increased 7- and 4-fold, 72 hr after isolation, but the gene expression of MEF-2c and Oct-1 was reduced to 10% and 70%, at day 3 in culture. We show atrial natriuretic peptide and brain natriuretic peptide gene expression to be maximal 24 and 72 hr after isolation, the level being 3- and 2-fold, when compared with freshly isolated cells. The gene expression of α- and β-myosin heavy chain was reduced to approximately 30% at day 3 in culture and similar observations were made for α-cardiac and α-skeletal actin, which declined to approximately 20% and 10% of control values, 72 hr after isolation. BDM prevented at the transcriptional level enhanced expression of markers for stress and metabolic deregulation, and the activities of lactate dehydrogenase and creatine kinase were highly significantly reduced. Similar results were obtained when explanted hearts were stored in BDM-containing organ preservation solution.**

Conclusions. **Preservation of metabolic function in donor organs is of critical importance in transplantation medicine, and we show gene markers for stress and metabolic deregulation in cultures of cardiomyocytes and explanted hearts to be significantly reduced by BDM. Reprogramming of gene expression of nu-**

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clear transcription factors and downstream target genes may prolong the acceptable storage time between explantation and transplantation.

Butanedione monoxime (BDM) is a known reversible myosin ATPase inhibitor (*1*) and is currently evaluated as an additive in solutions to preserve hearts for transplantation (*2–5*). Best results for long-term myocardial preservation are achieved at 30 mM concentrations and are linked to Na^+ channel blockade, Na^+,H^+ exchange inhibition, Na^+,K^+ $2Cl^-$ co-transport inhibition, and calcium desensitization (*6*). The mode of action for BDM may lead to cytoprotection in ischemic cardiomyocytes, and, in view of its favorable pharmacological properties in organ preservation, we wanted to investigate the effects of BDM on cardiomyocyte-specific gene expression. We thus investigated the expression of cardiomyocyte-specific transcription factor and of downstream target genes, which signal metabolic deregulation and are markers for stress and pathological processes.

The rational choice for the selection of the genes studied herein is based on their recently discovered role in molecular cardiology and is briefly summarized below.

It is now established that pathological changes in heart tissue are linked to changes in the gene expression pattern of a number of genes and indeed, during hypertrophy fetal isoforms of β-myosin heavy chain (β-MHC) and α-skeletal actin are up-regulated, whereas the corresponding adult isoforms α-MHC and α-cardiac actin are down-regulated (*7, 8*). Cellular stress and metabolic deregulation in cardiomyocytes fosters the expression of the 28-amino acid polypeptide atrial natriuretic peptide (ANP) and the brain-type natriuretic peptide (BNP), which are mainly secreted in atrial and ventricular tissue, respectively, to cause decreased blood pressure by vasodilatation and natriuresis, all of which are up-regulated in the heart in response to hypertrophic signals (*9, 10*). Whether β-MHC and α-skeletal actin as well as ANP and BNP are coordinately regulated in pathological processes remains uncertain, but common binding sites for several transcription factors of the above-named marker genes are suggestive for a coordinate response during cardiac remodeling and disease.

We thus investigated the gene expression of the transcription factors GATA4, MEF-2c, Nkx2.5, and Oct-1, all of which have established DNA consensus binding sites in the promotor regions of the target genes ANP, BNP, α-MHC, β-MHC, cardiac troponin I, and cardiac α-actin (*11–14*). There is also growing evidence that transcription factors are of critical importance in the onset of human heart failure, as well as congenital heart disease (*15–18*); taken collectively, the cumulative evidence for their key role in pathogenesis provided the rational for the selection of the herein studied genes.

Overall, this study aims to evaluate the ability of BDM to prevent enhanced gene expression of certain nuclear transcription factors and downstream target genes, which signal stress and metabolic deregulation in cultures of adult cardiomyocytes and explanted hearts. Reprogramming of gene expression during donor organ storage may be of benefit in organ preservation and thus could prolong the acceptable time period between explantation and transplantation.

MATERIALS AND METHODS

Animals. All animal procedures described in this report were approved by the local authorities, and the investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication 85-23, revised 1996). Male Sprague-Dawley rats weighing 208±37 g were obtained from Charles River Laboratories (Sulzfeld, Germany). Food and water was given ad libitum.

Anesthesia. Rats were anesthetized with ketamine (anesthetic) and xylazine hydrochloride (muscle relaxant) with 0.1 ml of ketamine/100 g of body weight and 0.05 ml of xylazine hydrochloride/100 g of body weight. In addition, 2,000 international units of heparin were administered intraperitoneally before surgery.

Isolation of adult cardiomyocytes. The thorax was opened by surgical procedures, and the aorta ascendens was carefully prepared. The heart was perfused in situ with the washing solution for 1 min (Joklik medium [Biochrom, Berlin, Germany], 500 μM EDTA, 15 mM BDM). After the initial wash, the heart was swiftly removed and

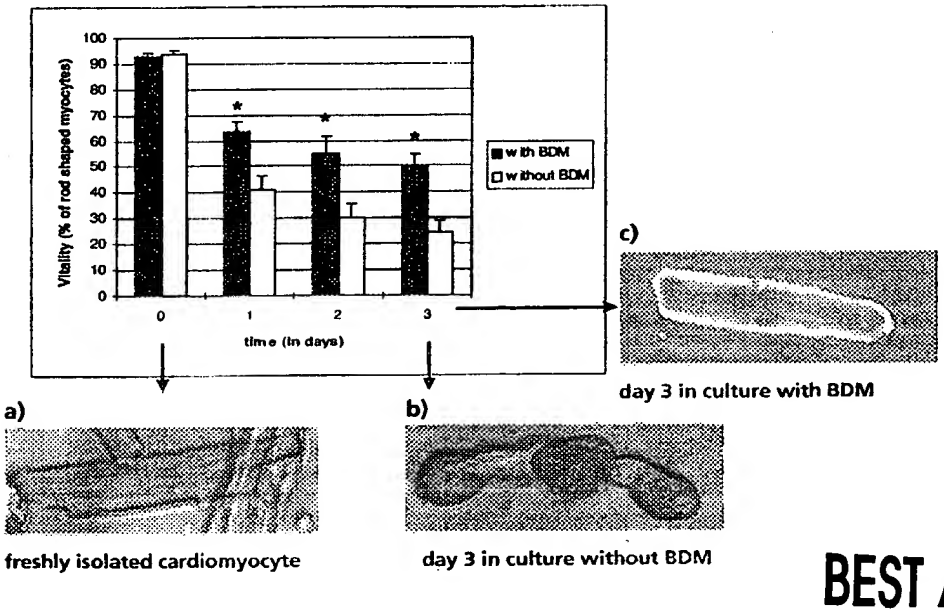

FIGURE 1. Viability of cardiomyocytes expressed as percentage of rod-shaped myocytes. (a) Freshly isolated cardiomyocyte. (b) Day 3 in culture without BDM. (c) Day 3 in culture with BDM. Data represent mean ± SEM of n=3 different isolations with approximately 2 million cells per culture dish.

mounted onto the perfusion apparatus. The washing solution was then pumped for 5 min through the heart; immediately thereafter, the enzyme solution for the isolation of cardiomyocytes (Joklik medium with 15 mM BDM, 1% bovine serum albumin, 20 μM calcium chloride, 1 mg/ml collagenase II) was pumped through the heart (Ismatec MCP pump; flow rate, 4 ml/min) for 25 min at 37°C. The heart tissue became tender at the end of the perfusion period and was transferred into a Petri dish containing 20 ml of the mincing solution (Joklik medium with 5% fetal calf serum). The heart was dissected with surgical scissors to obtain small pieces (<1 mm^3). Next, a cell suspension was prepared and filtered through a nylon mesh (Becton Dickinson, Heidelberg, Germany) of the size of 100 μm. For 3 min, the filtrate was centrifuged at 56 $\times g$ at room temperature. The resultant supernatant was discarded and an additional 30 ml of mincing solution was added to the cell pellet. The cell suspension was then transferred into a culture flask (75 cm^2; Corning Costar, Bodenheim, Germany) and put in the CO_2 incubator. After 15 min, a 2 mM calcium solution was cautiously added to the medium. The calcium concentration was then increased over 2 hr in a total of eight steps to obtain a final 1 mM concentration. Cells were also examined and photographed under phase contrast microscopy (Zeiss, Jena, Germany) to assess qualitatively and quantitatively the number of spherical and rod-shaped cells (Fig. 1).

The cells were counted in a Neubauer counting chamber (Hecht, Germany). Cells were cultured at 37°C at 5% CO_2 for 3 days. Purity of culture was >95% and was further improved by preplating cell suspension on plastic flasks for 2 hr as reported previously (*19*). Cardiomyocytes were cultured in the presence of BDM (15 mM), and the dose selection for the isolation and cultivation of heart muscle cells is based on literature findings where dose-response relationships with regard to organ preservation (*2–6*) and modulation of ion channels were investigated (see Table 1).

Studies with explanted hearts. The thorax was opened by standard surgical procedures, and the aorta ascendens was carefully prepared. The aorta was cannulated, and the heart was perfused (retrograde) in situ with Joklik medium. Perfusion was done for 1 min. The hearts were then removed and stored at 4°C for 6 hr in Joklik medium in the presence (n=3) or absence (n=3) of 15 mM BDM. Thereafter, biopsy materials were taken and quickly frozen in liquid nitrogen to await further analysis. For comparison, the gene expression of cardiomyocyte-specific transcription factors and downstream target genes was also studied in freshly explanted but unpreserved hearts (n=3).

RNA and cDNA. RNA was isolated from tissue samples and from cardiomyocytes shortly after isolation and also after 1 and 3 days of culture using the Spin-Vacuum total RNA isolation system (Promega, Mannheim, Germany) according to the manufacturer's recommendation. Quality of isolated RNA was checked using a 1.0% agarose gel. Total RNA (4 μg) from each sample was used for reverse transcription. RNA and random primer (Roche, Mannheim, Germany) were preheated for 10 min at 70°C. Then, 1×reverse transcriptase (RT)-avian myeloblastosis virus buffer, dNTPs (1.0 nM, Roche), 40 U of RNase inhibitor (Stratagene, Amsterdam, Netherlands), 20 U of RT-avian myeloblastosis virus (Promega, Mannheim, Germany) were added, and water treated with diethyl pyrocarbonate (Sigma, Diesenhofen, Germany) was added to a final volume of 20 μl. Next, reverse transcription was carried out for 60 min at 42°C and was stopped by heating to 95°C for 5 min. The resulting cDNA was frozen at −20°C until further experimentation.

Thermocycler RT-polymerase chain reaction (PCR). For the PCR amplification of cDNA, a 25-μl reaction mixture was prepared containing 10×polymerase reaction buffer, 1.5 mM $MgCl_2$, 0.4 nM dNTPs (Roche), 400 nM concentration of the 3'- and 5'-specific primer (synthesized by GIBCO, Karlsruhe, Germany), 1 U of *Taq* polymerase (Roche), and 1 μl of cDNA.

PCR reactions were carried out in a thermal cycler (Biometra) using melting, annealing, and extension cycling conditions as described in Table 1 (*23–35*). PCR products were separated using a 1.8% agarose gel, stained with ethidium bromide, and photographed on a transilluminator (see Fig. 2).

Real time semiquantitative RT-PCR. cDNA was diluted 1:10 with nuclease-free water, and 1 μl of the diluted cDNA was added to the

TABLE 1. Modulation of ion channels by BDM

Ref.	Species and Cell Type	Concentration of BDM	Ion Channel	Results
23	Canine cardiomyocytes	5 mM	Ca^{2+}	Excitation-contraction is suppressed from 5.9 to 3.4 mmHg
24	Canine cardiomyocytes	1–10 mM	Ca^{2+}	BDM activated cardiac and skeletal release channels
25	Guinea pig cardiomyocytes	12 mM	Ca^{2+}	BDM had no effect on current amplitude in the presence of trypsin, but decreased the time constant of slow inactivation to control values
26	Frog skeletal muscle cells	10–30 mM	Ca^{2+}	BDM suppresses Ca^{2+} release flux and intramembranous charge movement
27	Canine ventricular trabeculae	10 mM	Not known	BDM reduces peak isometric force to 10% and shortened action potential from 198 to 146 ms
28	*Xenopus* oocytes	10 mM	Ca^{2+}	IC_{50} 16 mM, BDM produced 36% inhibition of Ca^{2+} channel
29	Rabbit atrioventricular node	5–20 mM	AV node	Intra-atrial delay (50%) at 20 mM BDM
30	Guinea pig cardiomyocytes	20 mM	Ca^{2+}	BDM promotes voltage-dependent inactivation of L-type Ca^{2+} channels
31	Rat cardiomyocytes	5–200 mg/kg	Ca^{2+}	IC_{50} 17–29 mM; BDM lowers blood pressure in rats
32	Mouse pancreatic cells	10–30 mM	K^+	KI_{50} 15 mM
33	Rat ganglion neurons	10–30 mM	Ca^{2+}	IC_{50} 18.3 mM
34	Guinea pig cardiomyocytes	10–30 mM	Ca^{2+}	IC_{50} 5.8 mM; reduction of duration of action potential
35	Guinea pig cardiomyocytes	12–20 mM	Ca^{2+}	BDM reduced open propability of single cardiac L-type Ca^{2+} channels

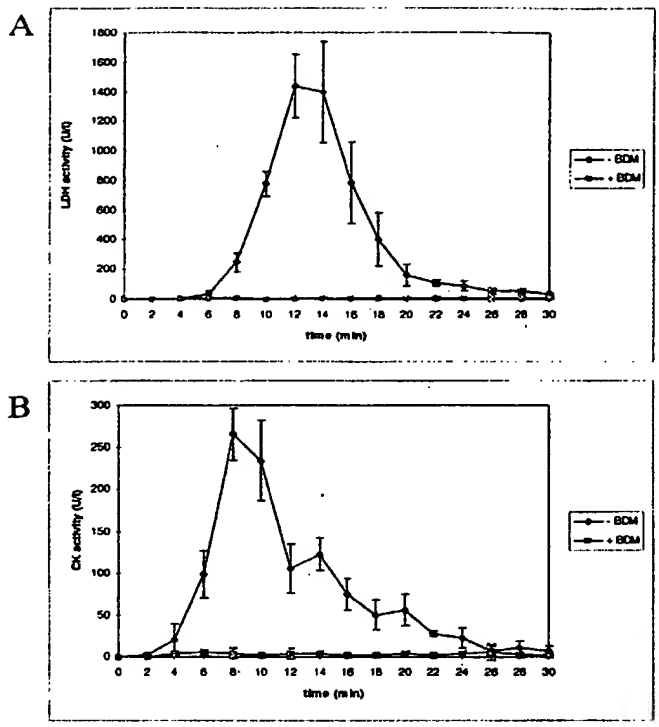

FIGURE 2. (A) LDH activity during isolation of cardiomyocytes. (B) CK activities during isolation of cardiomyocytes.

Lightcycler-Mastermix (0.5 μM specific primer, 3 mM $MgCl_2$, and 2 μl of Master-SYBR-Green [Roche]). This reaction mixture was filled up with water to a final volume of 20 μl. All reactions were done on a Lightcycler (Roche).

After an initial denaturation step at 95°C for 30s, the PCR reaction was done with annealing, extension and denaturation cycles described in Table 2. The PCR reaction was completed after a total of 35–45 cycles; at the end of each extension phase, fluorescence was observed and used for quantitative purposes. A melting-point analysis was carried out by heating the amplicon from 65°C to 95°C, and a characteristic melting-point curve was obtained for each product (data not shown).

In addition, a serial dilution experiment was carried out (n=3) with cDNA produced from cardiomyocyte total RNA using 1:10 dilution regiment to estimate the mRNA expression of genes. Control samples contained water instead of cDNA, and it should be noted that oligonucleotide dimers were produced in the PCR cocktail, but could easily be distinguished by the melting-point analysis.

Enzyme markers for membrane integrity. Lactate dehydrogenase (EC 1.1.1.27, U/L) and creatine kinase (EC 2.7.3.2, U/L) were measured as markers of metabolic stress and membrane integrity during isolation and in the media of cultured cardiomyocytes at 0, 24, and 72 hr using the Cobas Fara Roche device (Grenzach, Switzerland) and a protocol according to the manufacturer's recommendation.

Statistical analysis. Data represent mean ± standard deviation. The Wilcoxon signed-rank test was used, and differences were considered significant at $P<0.05$.

RESULTS

Cell Culture

For each isolation, approximately $11.9\pm0.3\times10^6$ cells/heart could be isolated with rod-shaped cells accounting for 92.7±1.8%. The purity of cardiomyocyte cultures was found to be >95% using standard procedures (see *Materials and Methods*) and phase contrast light microscopy. Freshly isolated cardiomyocytes were rod-shaped and had well-defined cross-striations (Fig. 1a). In the absence of BDM, cardiomyocytes developed cytoskeletal defects with loss of rod-shape morphology (Fig. 1b). In contrast, addition of BDM prevented the morphological change of cardiomyocytes as judged by the rod-shape morphology and the cross-striations at day 3 in culture (see Fig.1c). Addition of BDM resulted in an enhanced cell yield at all time points (Fig. 1), and the enzyme activities of lactate dehydrogenase (LDH) and creatine kinase (CK) were highly significantly reduced in the presence of BDM-containing isolation buffer (see Fig. 3).

Expression of Cardiomyocyte-specific Genes in Cell Cultures

ANP gene expression. In the presence of BDM, ANP gene expression was reduced to 40% and 50%, at 24 and 72 hr, respectively, when compared with freshly isolated cardiomyocytes. Cardiomyocyte cultures without BDM showed an approximate 2- to 3-fold increase in the stress marker ANP after 24 and 72 hr in culture (see Fig. 4).

BNP gene expression. Similar to ANP, the expression of BNP was greatest in the absence of BDM, the level being approximately 4-fold at 24 hr after isolation, but declined to an approximate 2-fold level against controls by 72 hr after isolation. With BDM, BNP gene expression was similar to controls (24 hr), but declined to 60% at 72 hr after isolation.

α-MHC gene expression. As with others, α-MHC gene expression was set to 100% for freshly isolated cardiomyocytes. Its expression was reduced to 40% and 10% in controls, at 24 and 72 hr after isolation, respectively. As shown in Figure 5, the gene expression differed between control and treatment groups as BDM attenuated the rapid decline in mRNA expression of α-MHC 24 and 72 hr after isolation.

β-MHC gene expression. BDM prevented the initial increase in β-MHC expression, the level being approximately 50% of control values (freshly isolated cardiomyocytes) 24 hr after isolation. As shown in Figure 4, cultures lacking BDM resulted in a 2-fold increase in β-MHC expression 24 hr after isolation, but declined to approximately 70% thereafter (72 hr).

α-Cardiac actin gene expression. When compared with freshly isolated cardiomyocytes (control values), treatment with BDM prevented rapid decline of α-cardiac actin gene expression (see Fig. 5). At day 3 in culture, however, expression levels were approximately 40% and 20% of the control and the treatment group, respectively.

α-Skeletal actin gene expression. mRNA expression was greatest in cultures without BDM 24 hr after isolation. Thereafter, neither the treatment group nor the control group differed in α-skeletal actin gene expression. Moreover, when compared with freshly isolated cardiomyocytes, a dramatic reduction in the mRNA expression could be observed, at day 3 in culture, the values being approximately 10% in the BDM and the control group (see Fig. 5).

Gene Expression of Nuclear Transcription Factors in Cell Cultures

GATA-4 gene expression. As shown in Figure 4, mRNA transcripts in controls increased 5- and 7-fold by 24 and 72

TABLE 2. Oligonucleotides used in RT-PCR[a]

Accession No.	Gene	Forward Primer	Reverse Primer	Product Length (bp)	Thermal Cycler Program	Extension Time (s)	Melting temperature (°C)
M60731	ANP	GCCGCTAGAAGATGAGGTCA	GGGCTCCAATCCTGTCAATC	269	1	12	90.6
M25297	BNP	TCTGCTCCTGCTTTTCCTTA	GAACTATGTGCCATCTTGGA	258	1	11	85.8
X15938	α-MHC	GGAAGAGCGAGCGGCGCATCAAGG	CTGCTGGACAGGTTATTCCTCA	304	2	13	90.9
X15939	β-MHC	GCCAACACCAACCTGTCCAAGTTC	TTCAAAGGCTCCAGGTCTCAGGGC	202	2	9	89.9
X80130	Cardial α-actin	ACTCCTATGTAGGTGACGAGGC	GACGTTATGAGTCACACCGTCG	337	3	15	89.4
V01218	Skeletal α-actin	ATCTCACGTTCAGCTGTGGTCA	ACCACCGGCATCGTGTTGGAT	182	3	8	88.3
AA955670	MEF2c	CCGATGCAGACGATTCAGTAG	GTGTCACACCAGGAGACATAC	260	4	11	85.6
L22761	GATA4	AGCAAGGACTAGGCACCTCTAGC	ATAGCCAGGCTTTGGTACATCGC	410	4	17	88.1
AF006664	Nkx2.5,	CACGCCTTCTCAGTCAAAGAC	AGGTACCGCTGTTGCTTGAAGC	461	4	19	89.2
U17013	Oct-1	CAATTGCAACAGCTTCAGCAGC	CATAGCAAGCCCAACATCACC	407	4	17	89.1
X02231	GAPDH	CGCCAAGGTCATCCATGA	TCAGTGAGCCCAGGATG	353	4	15	89.4

[a] (PCR programs: program 1: 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, 29 cycles; program 2: 60 s at 94°C, 60 s at 65°C, 90 s at 72°C, 28 cycles; program 3: 60 s at 94°C, 60 s at 55°C, 60 s at 72°C, 29 cycles; program 4: 45 s at 94°C, 45 s at 58°C, 60 s at 72°C, 28 cycles. All programs are initiated with a 2-min denaturation step and stopped with a 7-min final elongation step.)

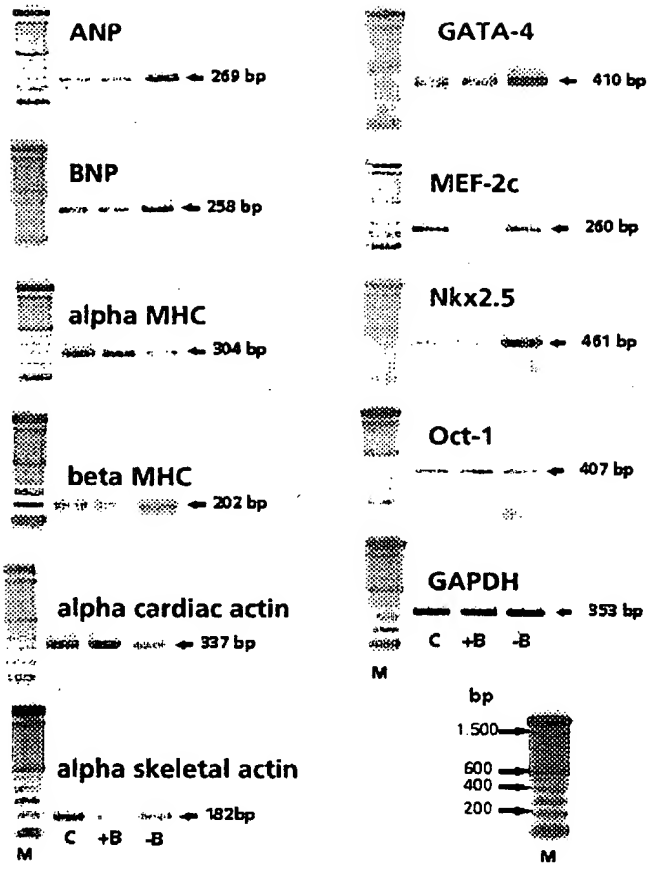

FIGURE 3. mRNA expression of genes ANP, BNP, α-MHC, β-MHC, α-cardiac actin, α-skeletal actin, and the transcription factors Gata-4, Mef-2c, Nkx2.5, Oct-1, and GAPDH in cultures of cardiomyocytes. M, molecular DNA weight marker; C, control; B, cultivated in the presence (+) or absence (−) of 15 mM BDM).

hr, respectively. In the BDM treatment group, the gene expression was similar to that of freshly isolated cells 24 hr after isolation, but declined to approximately 20% at day 3 in culture.

MEF-2c gene expression. In the BDM treatment group and when compared with freshly isolated cells, MEF-2c expression was reduced to approximately 20% and 10% at 24 and 72 hr after isolation, respectively. In controls, MEF2c levels were similar to freshly isolated cells at 24 hr after isolation, but declined thereafter to approximately 30%. Similar to the expression of the target genes α-cardiac actin and α-skeletal actin, the expression of MEF-2c declined rapidly after isolation and cultivation of cardiomyocytes (see Fig. 5).

Nkx2.5 gene expression. When compared with freshly isolated cardiomyocytes, the mRNA expression of Nkx2.5 increased 3-fold by 24 and 72 hr after isolation. This increase in Nkx2.5 expression was restricted to cultures lacking BDM. As shown in Fig. 4, gene expression of controls was almost identical to those treated with BDM (24 hr), but declined thereafter to about 50% of control values at 72 hr after

isolation. Noticeably, the gene expression pattern of ANP and BNP was similar to that of Nkx2.5.

Oct-1 gene expression The expression of Oct-1 did not differ between the control and the BDM treatment group at 24 hr after isolation. With the BDM treatment group, expression of Oct-1 declined over time to approximately 60% of control values (72 hr). In contrast, cultures lacking BDM Oct-1 mRNA levels were expressed close to control values, at 72 hr after isolation (see Fig. 5).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The gene expression of GAPDH did not differ between the control and BDM treatment group during the entire cultivation period (see Fig. 6).

Studies with Explanted Hearts

Figure 7 depicts the gene expression profiles in the various treatment groups. There was little difference in the mRNA expression profiles of ANP, BNP, β-MHC, GATA4, Nkx2.5, and OCT1, when freshly explanted and shock-frozen hearts (unpreserved) were compared with hearts preserved in Joklik medium containing 15 mM BDM. In contrast, omission of BDM resulted in significantly elevated gene expressions of β-MHC, GATA4, Nkx2.5, MEF2c, and OCT1. Thus, BDM alleviates the rapid decline in α-cardiac actin and α-MHC gene expression, but the opposite was seen in the case of MEF2c mRNA expression.

LDH and CK Enzyme Activity in Cultured Cardiomyocytes

Immediately after cell isolation, the levels of LDH and CK activities were 92.7±10.4 U/L and 13.3±3.3 U/L, respectively. Treatment of cell cultures with 15 mM BDM resulted in significant reductions in LDH and CK activities, by 24 and 72 hr after isolation and cultivation (see Fig. 8).

DISCUSSION

BDM has been studied extensively for its effects on electrical conductivity and, in particular, modulation of the L-type Ca^{2+} channel (see Table 1), but its mode of action at the transcriptional level, e.g., gene expression of Nkx2.5, GATA4, and downstream target genes, was until now unknown. There is conflicting information on IC_{50} values for the L-type Ca^{2+} channel blockade, with experimental values ranging from 5.8 to 29 mM. A representative selection of studies reporting IC_{50} values is given in Table 1. We did not attempt to correlate the gene expression data with electrophysiological measurements, as we aimed to study the gene expression of transcription factors and of downstream target genes in the presence of BDM, but we wish to point out that the rationale for the dose selection is purely based on its use in transplantation medicine (best results are obtained at 30 mM). However, in view of BDM's ability to inhibit ion channels (IC_{50} ranges between 6 and 30 mM), we selected a dose level halfway between its therapeutic dose in heart transplantation and its probable IC_{50} (range, 6-30 mM; see also Table 1).

Our study point to a similar expression of ANP, BNP, and β-MHC, and the transcription factors GATA-4 and Nkx2.5 followed a similar expression pattern in control and treated cell cultures, as well as in explanted and stored hearts. The expression levels of α-MHC, α-skeletal actin, and α-cardiac actin were similar to that of MEF2c, and this may or may not be the result of a co-ordinate genomic response.

We selected the transcription factors GATA-4, MEF-2c, Nkx2.5, and Oct-1 for their known and established role in the gene regulation of ANP, BNP, α-MHC, β-MHC, and cardiac α-actin, as reported by others (*11*, *13*, *14*). The latter downstream target genes are also considered to be good diagnostic markers in the onset of human heart failure as well as congenital heart disease (*7–10*). We were surprised that the gene expression pattern in our cardiomyocyte culture was similar to that of diseased patients, but we wish to emphasize that further experimental validation is needed to establish a causal relationship between gene expression patterns in cultures of cardiomyocytes and cardiac disease.

We thus investigated the gene expression profile in explanted hearts stored for 6 hr at 4°C and compared our findings with that of freshly explanted but shock-frozen hearts. We can clearly demonstrate that BDM results in reprogramming of gene expression in explanted hearts, even though this change was less pronounced when compared with the gene expression in cultured cardiomyocytes. This validates, at least in part, the change in gene expression data observed in cultures of cardiomyocytes, but we wish to emphasize that Joklik medium is not an established organ preservation solution in transplantation medicine. Nevertheless, for experimental reasons and to obtain valid comparisons, we needed to store explanted hearts in the same medium as our cultures of cardiomyocytes.

Reprogramming of gene expression will also translate into pharmacotherapy of cardiac disease, and our findings could be of considerable value for the development of new pharmacotherapeutic approaches, by targeting gene expression of transcription factors.

In the case of human heart failure, α-MHC gene expression is dramatically reduced, but its counterpart, β-MHC, is increased (*10*). There is also evidence that α-skeletal actin is increased during human heart failure, but the expression of α-cardiac actin is down-regulated (*7*). Evidence suggests that ANP and BNP gene expression are increased in the onset and progression of human heart failure (*9*). Little knowledge is, however, available from clinical investigations, when transcription factors, regulating these genes, are being assessed, but it is most likely that the transcription factors explored in our investigation are of critical importance in the regulation of the marker genes studied herein. Indeed, GATA-4 regulates, at least in part, ANP, BNP, α-MHC, and β-MHC (*14*), and we show for this particular transcription factor an approximate 7-fold increase when compared with controls (freshly isolated cardiomyocytes).

Expression of α-MHC differed from ANP, BNP, and β-MHC, probably as a result of different and, as yet, unknown interaction of transcription factor proteins and the regulatory elements of the above mentioned genes. In our experiments, we showed that β-MHC transcript levels were elevated in the absence of any treatment, whereas α-skeletal actin was significantly reduced to 20% of control values at 24 hr after isolation.

As shown in this study, Nkx2.5 was increased during cultivation of cardiomyocytes in cultures lacking BDM; likewise, the target gene regulated by this transcription factor, namely, ANP, was also elevated in the same treatment group. It is noteworthy that Nkx2.5, ANP, and BNP followed

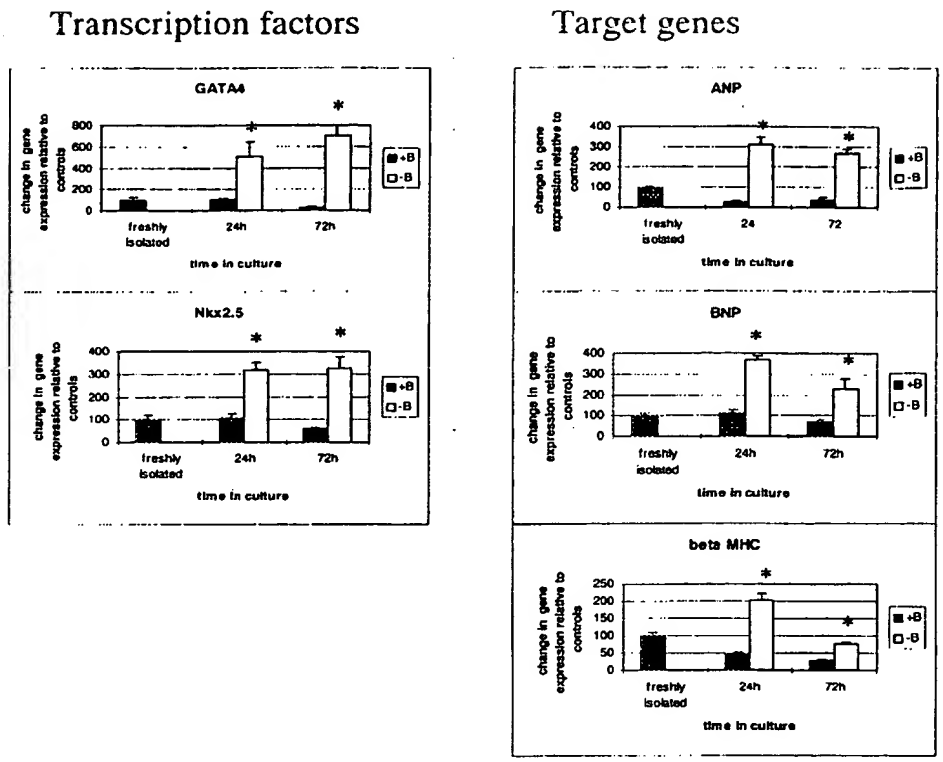

FIGURE 4. Semiquantitative real-time RT-PCR of transcription factors and target genes in cultures of cardiomyocytes. The results obtained with freshly isolated cells were set to 100%. The relative expression of GATA4, NKX2.5, ANP, BNP, and β-MHC is shown. B, cultured in the presence of 15 mM BDM. Data represent mean ± SEM of three individual cell culture experiments with approximately 2 million cells per culture dish (*, $P<0.05$).

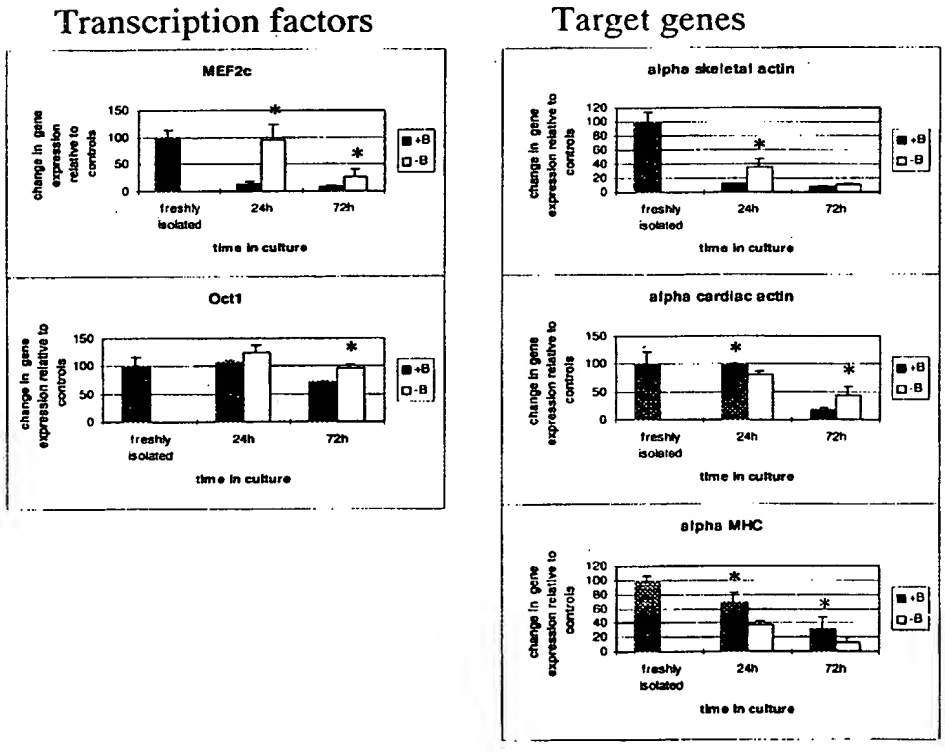

FIGURE 5. Semiquantitative real-time RT-PCR of transcription factors and target genes in cultures of cardiomyocytes. The results obtained with freshly isolated cells were set to 100%. The relative expression of MEF2c, Oct1, α-skeletal actin, α-cardiac actin, and α-MHC is shown. B, cultured in the presence of 15 mM BDM. Data represent mean ± SEM of three individual cell culture experiments with approximately 2 million cells per culture dish (*, $P<0.05$).

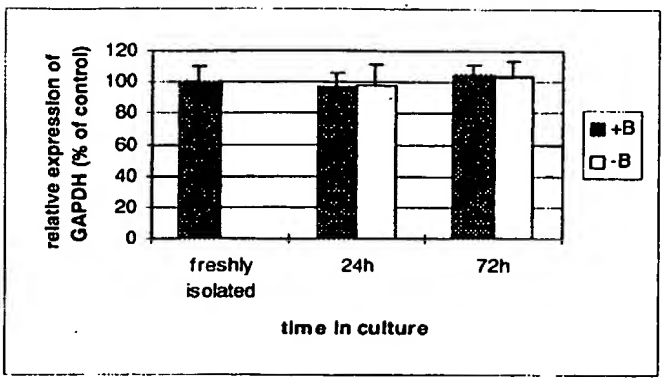

FIGURE 6. Semiquantitative real-time RT-PCR of GAPDH. The results obtained with freshly isolated cells were set to 100%. The relative expression of GAPDH is shown. B, cultured in the presence of 15 mM BDM. Data represent mean ± SEM of three individual cell culture experiments with approximately 2 million cells per culture dish (*, $P<0.05$).

a similar expression pattern during the entire cultivation period, presumably as the Nkx2.5 protein regulates the expression of the latter gene (*20*). Corroborative evidence for a co-ordinate expression of α-MHC and MEF-2c was obtained in the course of our study, and Lee et al. (*11*) have shown the co-ordinate regulation of α-MHC by MEF-2c. Gene expression of α-MHC is down-regulated in human heart failure (*10*), and we show a similar pattern for MEF-2c, but we emphasize that further analysis of all MEF-2 and Oct-1 binding sites is needed (genomic in vivo footprinting) to explore the mechanism of gene activation of myosin heavy chain (*12*).

From a clinical point of view, gene markers are of great utility as prognostic disease markers and are important in the early discovery of pathogenic events. Worldwide, the search for suitable "marker genes or gene biomarkers" continues. It is now accepted that reprogramming of gene expression in the adult myocardium is one of the critical events in disease, and, as already pointed out, enhanced expression of fetal isoforms like β-myosin heavy chain and α-skeletal actin are up-regulated, whereas the corresponding adult isoforms α-myosin heavy chain and α-cardiac actin genes are down-regulated in hypertrophy of the myocard (*7–10*). This process of tissue remodeling may be viewed as the selective increase or repression of cardiac gene expression.

We observed cardiomyocytes to develop pseudo-podium-like structures in culture, if BDM was not added to the culture medium (data not shown), and it is significant that, in such cultures, α-MHC gene expression is dramatically reduced, but this decline could be prevented, once again, if BDM was added to the culture medium. The relationship between morphological alteration and selective gene expression patterns warrants further studies, and, interestingly, our findings correlate well with results from a clinical study. Indeed, when left ventricular biopsies from a total of 21 patients with clinically diagnosed chronic hibernating myocardium were examined by light and electron microscopy, a mean of 27% of cardiomyocytes were found to be cellularly dedifferentiated rather than degenerated (*21*) and cytoskeletal damage of cardiomyocytes was considered to be a consequence of myocardial ischemia leading to subsarcolemmal blebs and breaks in the plasma membranes of cardiomyocytes (*22*). This correlates well with the cytoskeletal defects observed in morphologically dedifferentiated cardiomyocyte cultures (data not shown).

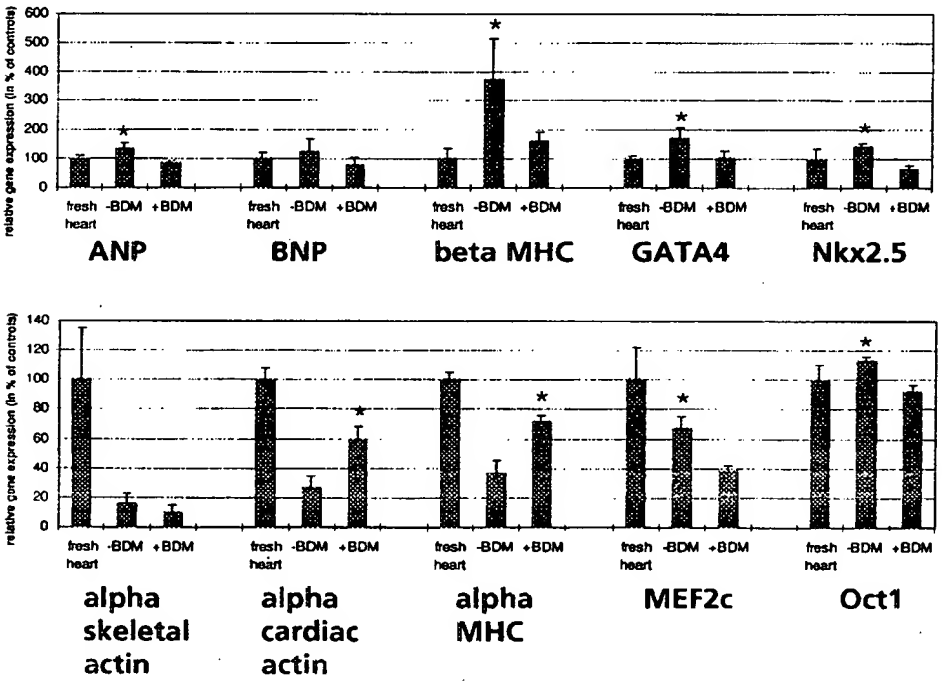

FIGURE 7. Semiquantitative real-time PCR of transcription factors and downstream target genes in explanted hearts. The results obtained with freshly explanted hearts were set to 100%. Data represent mean ± SEM of three individual explanted hearts (*, $P<0.05$).

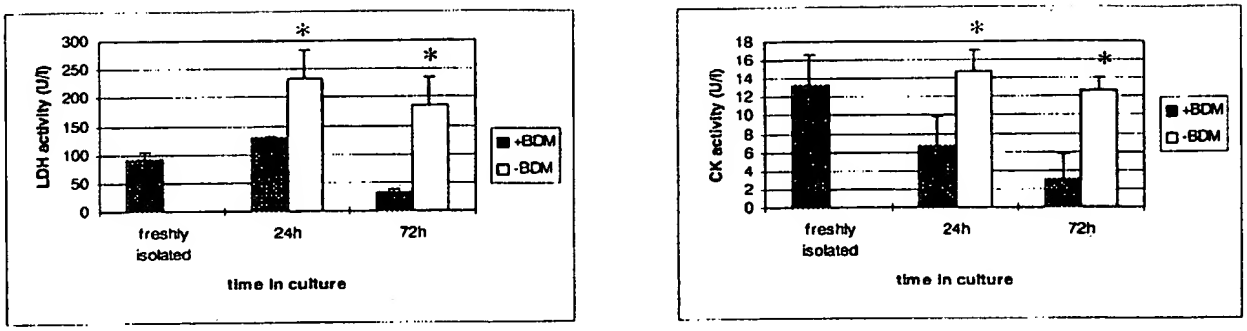

FIGURE 8. Lactate dehydrogenase and creatine kinase activity after cell isolation and 24 and 72 hr after isolation. Data represent mean ± SEM of three individual cell culture experiments with approximately 2 million cells per culture dish (*, $P<0.05$).

In conclusion, our investigations provide evidence for a reprogramming of gene expression of transcription factors and target genes in cultures of adult cardiomyocytes and in explanted hearts. This process can be modulated by the myosin ATPase inhibitor BDM, which warrants further studies into its use in transplantation medicine.

REFERENCES

1. Cramer LP, Mitchison TJ. Myosin is involved in postmitotic cell spreading. J Cell Biol 1995; 131: 179.
2. Zhang J, Furukawa RD, Fremes SE, Mickle DA, Weisel RD. Effects of butanedione monoxime and temperature on prolonged cardiac storage. J Membr Biol 1997; 1: 189.
3. Vahl CF, Bonz À, Hagl C, Hagl, S. Reversible desensitization of the myocardial contractile apparatus for calcium: a new concept for improving tolerance to cold ischemia in human myocardium? Eur J Cardiothorac Surg 1994; 8: 370.
4. Stowe DF, Boban M, Graf BM, Kampine JP, Bosnjak ZJ. Contraction uncoupling with butanedione monoxime versus low calcium or high potassium solutions on flow and contractile function of isolated hearts after prolonged hypothermic perfusion. Circulation 1994; 89: 2412.
5. Stringham JC, Paulsen KL, Southard JH, Fields BL, Belzer FO. Improved myocardial ischemic tolerance by contractile inhibition with 2,3-butanedione monoxime. Ann Thorac Surg 1992; 54: 852.
6. Snabaitis AK, Chambers D. Long-term myocardial preservation: beneficial and additive effects of polarized arrest (Na^+-channel blockade), Na^+/H^+-exchange inhibition, and $Na^+/K^+/2Cl^-$cotransport inhibition combined with calcium desensitization. Transplantation 1999; 27: 1444.
7. Schwartz K, Chassagne C, Boheler KR. The molecular biology of heart failure. J Am Coll Cardiol 1993; 22: 30A.
8. Nakao K, Monobe W, Roden R, Bristow MR, Leinwand LA. Myosin heavy chain gene expression in human heart failure. J Clin Invest 1997; 100: 2362.
9. Yoshimura M, Yasue H, Okumura K, et al. Different secretion patterns of atrial natriuretic peptide and brain natriuretic peptide in patients with congestive heart failure. Circulation 1993; 87: 464.
10. Lowes BD, Minobe W, Abraham WT, et al. Changes in gene expression in the intact human heart: downregulation of α-myosin heavy chain in hypertrophied, failing ventricular myocardium. J Clin Invest 1997; 100: 2315.
11. Lee Y, Nadal GB, Mahdavi V, Izumo S. Myocyte-specific enhancer factor 2 and thyroid hormone receptor associate and synergistically activate the α-cardiac myosin heavy-chain gene. Mol Cell Biol 1997; 17: 2745.
12. Lakich MM, Diagana TT, North DL, Whalen RG. MEF-2 and Oct-1 bind to two homologous promoter sequence elements and participate in the expression of a skeletal muscle-specific gene. J Biol Chem 1998; 273: 15217.
13. Sepulveda JL, Belaguli N, Nigam V, et al. GATA-4 and Nkx2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. Mol Cell Biol 1998; 18: 3405.
14. Charron F, Paradis P, Bonchain O, Nemer G, Nemer M. Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression. Mol Cell Biol 1999; 19: 4355.
15. Durand JB, Bachinski LL, Bieling LC, et al. Localization of a gene responsible for familial dilated cardiomyopathy to chromosome 1q32. Circulation 1995; 92: 3387.
16. Molkentin JD, Lu JR, Antos CL, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998; 93: 215.
17. Schott JJ, Benson DW, Basson CT, et al. Congenital heart disease caused by mutations in the transcription factor Nkx2–5. Science 1998; 281: 108.
18. Pehlivan T, Pober BR, Brueckner M, et al. GATA4 haploinsufficiency in patients with interstitial deletion of chromosome region 8p23.1 and congenital heart disease. Am J Med Genet 1999; 83: 201.
19. Chajek T, Stein O, Stein Y. Rat heart in culture as a tool to elucidate the cellular origin of lipoprotein lipase. Biochim Biophys Acta 1977; 488: 140.
20. Tanaka M, Chen Z, Bartunkova S, Yamasaki N, Izumo S. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. Development 1999; 126: 1269.
21. Ausma J, Cleutjens J, Thone F, et al. Chronic hibernating myocardium: interstitial changes. Mol Cell Biochem 1995; 147, 35.
22. Steenbergen C, Hill ML, Jennings RB. Cytoskeletal damage during myocardial ischemia: changes in vinculin immunofluorescence staining during total in vitro ischemia in canine heart. Circ Res 1987; 60: 478.
23. Takasago T, Goto Y, Kawaguchi O, et al. 2,3-Butanedione monoxime suppresses excitation-contraction coupling in the canine blood-perfused left ventricle. Jpn J Physiol 1997; 47: 205.
24. Tripathy A, Xu L, Pasek DA, Meissner G. Effects of 2,3-butanedione 2-monoxime on Ca2+ release channels (ryanodine receptors) of cardiac and skeletal muscle. J Membr Biol 1999; 1: 189.
25. Dooley PC, Hancox JC, Chapman RA. Effect of phosphorylation on L-type calcium current in ventricular myocytes dialysed with proteolytic enzymes. Clin Exp Pharmacol Physiol 1999; 26: 109.
26. De Armas R, Gonzalez S, Brum G, Pizarro G. Effects of 2,3-butanedione monoxime on excitation-contraction coupling in

frog twitch fibres. J Muscle Res Cell Motil 1998; 1998: 19: 961.
27. Biermann M, Rubart M, Moreno A, et al. Differential effects of cytochalasin D and 2,3 butanedione monoxime on isometric twitch force and transmembrane action potential in isolated ventricular muscle: implications for optical measurements of cardiac repolarization. J Cardiovasc Electrophysiol 1998; 9: 1348.
28. Allen TJ, Mikala G, Wu X, Dolphin AC. Effects of 2,3-butanedione monoxime (BDM) on calcium channels expressed in *Xenopus* oocytes. J Physiol (Lond) 1998; 1: 1.
29. Cheng Y, Mowrey K, Efimov IR, et al. Effects of 2,3-butanedione monoxime on atrial-atrioventricular nodal conduction in isolated rabbit heart. J Cardiovasc Electrophysiol 1997; 8: 790.
30. Ferreira G, Artigas P, Pizarro G, Brum G. Butanedione monoxime promotes voltage -dependent inactivation of L-type calcium channels in heart: effects on gating currents. J Mol Cell Cardiol 1997; 29: 777.
31. Xiao YF, McArdle JJ. Effects of 2,3-butanedione monoxime on blood pressure, myocardial Ca^{2+} currents, and action potentials of rats. Am J Hypertens 1995; 8: 1232.
32. Smith PA, Williams BA, Ashcroft FM. Block of ATP-sensitive K^+ channels in isolated mouse pancreatic β-cells by 2,3-butanedione monoxime. Br J Pharmacol 1994; 112: 143.
33. Zhu Y, Ikeda SR. 2,3-Butanedione monoxime blockade of Ca^{2+} currents in adult rat sympathetic neurons does not involve "chemical phosphatase" activity. Neurosci Lett 1993; 155: 24.
34. Chapman RA. The effect of oximes on the dihydropyridine-sensitive Ca current of isolated guinea-pig ventricular myocytes. Pflugers Arch 1993; 422: 325.
35. Allen TJ, Chapman RA. The effect of a chemical phosphatase on single calcium channels and the inactivation of whole-cell calcium current from isolated guinea-pig ventricular myocytes. Pflugers Arch 1995; 430: 68.

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TRANSPLANTATION

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TRANSFERRIN RECEPTOR-MEDIATED GENE TRANSFER TO THE CORNEAL ENDOTHELIUM[1]

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Background. **The application of gene therapy to prevent allograft rejection requires the development of noninflammatory vectors. We have therefore investigated the use of a nonviral system, transferrin-mediated lipofection, to transfer genes into the cornea with the aim of preventing corneal graft rejection.**

Methods. **Rabbit and human corneas were cultured ex vivo and transfected with either lipofection alone or in conjunction with transferrin. The efficiency of transfection, localization, and kinetics of marker gene expression were determined. Strategies to increase gene expression, using chloroquine and EDTA, were investigated. In addition to a marker gene, a gene construct encoding viral interleukin 10 (vIL-10) was transfected and its functional effects were examined in vitro.**

Results. **Transferrin, liposome, and DNA were demonstrated to interact with each other, forming a complex. This complex was found to deliver genes selectively to the endothelium of corneas resulting in gene expression. Treatment of corneas with chloroquine and EDTA increased the transfection efficiency eightfold and threefold, respectively. We also demonstrated that constructs encoding vIL-10 could be delivered to the endothelium. Secreted vIL-10 was shown to be functionally active by inhibition of a mixed lymphocyte reaction.**

Conclusions. **Our data indicate that transferrin-mediated lipofection is a comparatively efficient nonviral method for delivering genes to the corneal endothelium. Its potential for use in preventing graft rejection is shown by the ability of this system to induce vIL-10 expression at secreted levels high enough to be functional.**

Although corneal transplants enjoy relative immune privilege (*1*), there is a significant allograft failure rate after

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